Original Article

Evaluation of Acute and Sub-Chronic Toxicity of *Lactobacillus rhamnosus* GG in Sprague-Dawley Rats

Vedam Venkata Kanthi Vaishnavi[1,2], Urmila Banik[3], Gokul Shankar Sabesan[4], Arun K. Adhikary[5], Subramani Parasuraman[6]

[1]Department of Oral Pathology, Faculty of Dentistry, AIMST University, Bedong, Kedah Darul Aman, Malaysia, [2]Department of Microbiology, Faculty of Medicine, AIMST University, Bedong, Kedah Darul Aman, Malaysia, [3]Faculty of Medicine, Nursing & Health Sciences, SEGi University, Kota Damansara, Malaysia, [4]Department of Microbiology, Faculty of Medicine, Manipal University College-Malaysia (MUCM), Malaysia, [5]International Medical School, Management and Science University, Shah Alam, Selangor, Malaysia, [6]Department of Pharmacology, Toxicology and Basic Health Sciences, Faculty of Pharmacy, AIMST University, Bedong, Kedah Darul Aman, Malaysia

Abstract

Background: Probiotic-based bacteriotherapy has emerged as a potentially effective strategy for preventing infectious diseases. *Lactobacillus* strains consumed as probiotics and the safety of these spp. has been questioned due to reported unexpected responses. Hence, the present study has been conducted to evaluate the acute and sub-chronic toxicity of *Lactobacillus rhamnosus* GG in Sprague-Dawley (SD) rats.

Materials and Methods: The acute and sub-chronic toxicity effect of *L. rhamnosus* is studied in rats as per the Organization for Economic Cooperation and Development (OECD), test guideline 423 and 407, respectively.

Results: In acute toxicity, *L. rhamnosus at* 1×10^7, 1×10^8, 1×10^9, and 1×10^{10} CFU/mL don't show any toxic signs. In sub-chronic toxicity, *L. rhamnosus* at 1×10^6,1×10^7 and 1×10^8 CFU/mL dosages showed dose-depended changes in biochemical and haematological parameters. In this study, one male and one female rat administered with 1×10^8 CFU/mL of *L. rhamnosus* showed mortality on days 16 and 26, respectively. The animals administered with *L. rhamnosus* showed no histological changes in the organs such as heart, liver and kidney.

Conclusion: *L. rhamnosus* exhibited mild-to-moderate toxic effects at the dose levels of 1×10^6 CFU/mL, 1×10^7 and 1×10^8 CFU/mL in rats.

Keywords: *Lactobacillus rhamnosus,* probiotics, toxicity

Address for correspondence: Dr. Vedam Venkata Kanthi Vaishnavi, Department of Oral Pathology, Faculty of Dentistry, AIMST University, Bedong, Kedah Darul Aman, Malaysia.
E-mail: vaishnavivedam@gmail.com
Submitted: 08-Jan-2024; **Revised:** 20-Feb-2024; **Accepted:** 06-Mar-2024; **Published:** 28-Dec-2024

Introduction

Probiotics have been considered an upcoming strategy in the prevention of various local and systemic infections in the human body. *"Probiotics"* are live microorganisms which when provided in appropriate quantities have beneficial health effects on the host.[1-3] Bacteriotherapy with these probiotics is a promising and cost-effective approach for preventing diseases.[4] In this method, the host is given naturally existing helpful bacteria to help balance the equilibrium in between harmful and beneficial bacteria to improve the patient's oral and systemic health. This balance of multiple species of microorganisms (bacteria, fungi, protozoa, and archaea) plays a major role in maintaining the body's immune response. Among them probiotic strains unique to the genera *Lactobacillus, Bifidobacterium,* and *Streptococcus* have been widely prescribed for therapeutic uses.[5] These probiotics particularly act *via* two basic routes (direct and indirect mechanisms) in the human body. Probiotic bacteria may produce either antimicrobial

Access this article online

Quick Response Code:

Website: www.advbiores.net

DOI: 10.4103/abr.abr_13_24

How to cite this article: Vaishnavi VVK, Banik U, Sabesan GS, Adhikary AK, Parasuraman S. Evaluation of acute and sub-chronic toxicity of *Lactobacillus rhamnosus* GG in sprague-dawley rats. Adv Biomed Res 2024;13:126.

substances directly or anti-inflammatory substances indirectly. Other less common changes include alteration of humoral and cell-mediated immune responses and enzymatic reactions.

Lactobacillus strains are commonly used probiotic and known as "Generally Recognized as Safe (GRAS),"[6] their safety remains a subject of controversy due to reported unexpected reactions like *Lactobacillus*-associated local and systemic effects. Ongoing studies in microbiome science is leading to the creation of innovative ways to treat healing illnesses and increase the host well-being supported by probiotics. Among these, probiotics are gaining major attention in improving overall oral health, abdominal disorders, urinary tract infections, and cancer-related issues.[7] Recently, apart from the promising research in medical field, several diseases like dental caries (decay), periodontal diseases (gum diseases), halitosis (bad breath), and various common fungal infections of the mouth have gained more attention in therapeutic research by using probiotics.

Although, *L. rhamnosus* is a well-known strain of bacteria commonly used in probiotic supplements and few dairy products. The studies have reported the incidences of adverse systemic reactions in the humans on consuming these probiotics.[8] As these probiotics are administered in live form for several diseases, every batch of the chosen probiotic strain ideally needs to be monitored using appropriate *in vitro* studies, animal studies, and clinical trials at regular intervals to avoid unwanted adverse reactions in the future.[9] Probiotics usage at prescribed dose levels is needed to improve human well-being. Probiotics are evaluated based on various parameters, such as the selection of appropriate dosage, administration strategy, clinical outcome and symptoms, duration of exposure, and body weight and systemic changes.[5] Of this, the provision of probiotics to consumers in a suitable dosage form parameter is considered as prime importance. This determination of the dosages for the use of probiotics can be selected by performing proper standardized methods of pharmacological evaluation using preclinical laboratory experiments and clinical trials. In the present study, safety assessment of *L. rhamnosus* strain using acute and sub-acute oral toxicity tests was conducted in Sprague-Dawley (SD) rats as per the Organization for Economic Cooperation and Development (OECD) test guideline (TG) 423 and 407, respectively.[10,11]

Materials and Methods

Lactobacillus rhamnosus

The *L. rhamnosus* (Family: Lactobacillaceae) ATCC 53103 was purchased from SPD Scientific (M) Sdn. Bhd. Malaysia and cultured in de Man, Rogosa, and Sharpe (MRS) broth (New England Biolabs, USA) and agar medium (Neogen, USA) at 37°C with 95% air and 5% CO_2 condition. Later, the culture was centrifuged at 5000 × g for 10 min at 4°C. The supernatant was disposed, and the cell pellets underwent three washes with deionized water. Following this, they were freeze-dried and stored at -20°C until needed. Fresh cell suspensions from the freeze-dried stocks were prepared regularly before administering them to the animals *via* gavage in MRS media.

Animals and housing

Healthy, adult, male, and female SD rats were obtained from the Central Animal House, AIMST University, Malaysia. The rats were maintained at standard laboratory conditions at room temperature (20–25° C and 60–65% relative humidity) with a 12 h light and 12 h dark cycle. The rats were given water and fed with a standard rodent pellet diet *ad libitum*. To reduce stress, the rats were acclimated to the lab environment for two weeks before the experiment. Prior approval was obtained from the AIMST University Human and Animal Ethics Committee (AUAEC/FOM/2021/02) to carry out the study. The study was conducted in compliance with the OECD TG 423 and 407, respectively.

Acute oral toxicity study

An acute oral toxicity effect of *L. rhamnosus* (suspended in MRS media) was studied according to the OECD TG 423 using a step-wise procedure.[10] Healthy female rats (three animals/dose) were used. The rats that were fasted overnight were orally administered with *L. rhamnosus* in increasing dose levels of 1×10^7, 1×10^8, 1×10^9, and 1×10^{10} CFU/mL, respectively. Animals were continuously monitored for 24 h to note the changes in behavioural and neurological functions. Later, the rats were observed for 14 days for mortality.

Sub-chronic oral toxicity study

The sub-chronic oral toxicity effect of *L. rhamnosus* (suspended in MRS media) study using 28 days repeated-dose toxicity study protocol as per OECD TG 407.[11] A total of forty-eight rats (180 ± 20 g) were used in this study. The animals were divided into four groups with twelve animals each (six male and six female/group) as follows:

Group I: Vehicle control

Group II: *L. rhamnosus* (1×10^6 CFU/mL; 1 mL/ day)

Group III: *L. rhamnosus* (1×10^7 CFU/mL; 1 mL/ day)

Group IV: *L. rhamnosus* (1×10^8 CFU/mL; 1 mL/ day)

The dose selection was based on acute toxicity study results. During the study, animal mortality (if any), changes in body weight (BW) were monitored at regular intervals. On the 14th and 28th day of the experiment, the blood samples were collected from retro-orbital plexus puncture under diethyl ether anesthesia in non-heparinized blood collection tubes and sodium ethylenediaminetetraacetic acid (EDTA) tubes and used for biochemical and haematological analysis, respectively.

Haematological parameters such as red blood cell count (RBC), hemoglobin (Hb), packed cell volume (PCV), mean corpuscular volume (MCV), mean corpuscular hemoglobin (MCH), mean corpuscular hemoglobin concentration (MCHC), red cell distribution width (RDW), total white blood cell count (WBC), differential WBC count, and platelets were analyzed

using an automated haematological analyzer (Sysmex XN-1000 hematology analyzer). Serum biochemical analysis was done at the laboratory using an automatic clinical chemistry analyzer (Roche cobas c502 analyser) for analyzing parameters such as glucose, total protein, albumin, globulin, total bilirubin, aspartate aminotransferase (AST), alanine aminotransferase (ALT), alkaline phosphatase (ALP), gamma-glutamyl transpeptidase (GGT), urea, uric acid, creatinine, calcium, corrected calcium and phosphate, total cholesterol (TC), triglyceride (TGL), high-density lipoprotein (HDL), low-density lipoprotein (LDL), and non-HDL cholesterol. The total cholesterol/HDL ratio was calculated mathematically. Both haematological and biochemical analyses were done by Innoquest Pathology Sdn. Bhd., Malaysia.

At the end of the study, the animals were euthanized by diethyl ether anesthesia and the organs such as brain, heart, lung, liver, spleen, kidney, adrenal, and gonadal organs were collected for organ weight analysis. Further, the organs such as the heart, liver, and kidney were fixed in 10% formalin, dehydrated for 16 h in an automatic tissue processor (Thermo Scientific Excelsior AS, USA), and embedded in paraffin wax using a paraffin embedding system (Sakura Tissue Tek Embedder, USA). Each sample was sliced into 5-µm-thick sections using a rotary microtome (Leica RM 2235, Japan) The sections were fixed on a glass slide, stained with hematoxylin and eosin (H&E), and examined under light microscopy.

Statistical analysis

All the data were presented as mean ± standard error mean (SEM). The data were analyzed using one-way analysis of variance (ANOVA) followed by Tukey's *post-hoc* test. A value of $P < 0.05$ was considered to be statistically significant.

Results

Acute oral toxicity study

In acute oral toxicity testing, the rats administered with 1×10^7, 1×10^8, 1×10^9, and 1×10^{10} CFU/mL of *L. rhamosus* did not show any mortality and untoward effects. There was no evidence of changes in the body weight, behavioural and neurological functions. At necropsy, the experimental rats were free from gross pathological changes. Further sub-chronic oral toxicity study was carried out using 1×10^6, 1×10^7, and 1×10^8 CFU/mL *L. rhamnosus* in rats.

Sub-chronic oral toxicity study

The signs of toxicity were monitored throughout the study in all the experimental groups. Furthermore, the rats administered with repeated doses of *L. rhamnosus* 1×10^6, 1×10^7, and 1×10^8 CFU/mL did not show any significant changes in the body weight, and feed consumption as compared with the control groups. The male rats administered with 1×10^8 CFU/mL *L. rhamnosus* showed mortality of one animal on day 16, and the female rats administered with 1×10^8 CFU/mL *L. rhamnosus* showed mortality of one animal on day 26.

i. Effect of *L. rhamnosus* on the body weight and relative organ weight changes

Throughout the study, the animals administered with different doses of *L. rhamnosus* did not show any significant changes in body weight when compared with that of the control. At the termination of the study, no changes in the gross pathology were noted in any of the experimental animals. The animals administered with *L. rhamnosus* exhibited no significant changes in the absolute and relative weights of brain, heart, lung, liver, spleen, kidney, adrenal, and gonadal organs as compared with the control group.

ii. Effect of *L. rhamnosus* on the biochemical parameters

In the biochemical analysis, the animals administered with *L. rhamnosus* exhibited noteworthy changes in the liver and kidney related parameters. On day 14, the male animals administered with 1×10^6, 1×10^7, and 1×10^8 CFU/mL *L. rhamnosus* did not show any significant alteration in the biochemical parameter when compared with that of the control group [Figure 1a]. Whereas female animals administered with 1×10^7 CFU/mL of *L. rhamnosus* showed a significant change in the levels of AST and ALT when compared with that of the control group; female animals administered with 1×10^8 CFU/mL of *L. rhamnosus* exhibited a significant increase in the levels of sodium, albumin, and globulin when compared with that of the control group [Figure 1b].

On day 28, the male animals administered with 1×10^7 CFU/mL of *L. rhamnosus* showed significant changes in the uric acid, albumin and potassium when compared with that of the control group; male animals administered with 1×10^8 CFU/mL of *L. rhamnosus* showed a significant change in the level potassium, uric acid and chloride when compared with that of the control group [Figure 2a]. Whereas female animals administered with 1×10^6 CFU/mL of *L. rhamnosus* showed a significant rise in the level of sodium and corrected calcium when compared with that of the control group; female animals administered with 1×10^7 CFU/mL of *L. rhamnosus* significant increase in the levels of sodium, urea, creatinine, corrected calcium, globulin, AST, ALT and decrease in the level of chloride when compared with that of the control group; female animals administered with 1×10^8 CFU/mL of *L. rhamosus* showed a notable increase in the level of sodium, uric acid, corrected calcium, AST, and ALT and decreased levels of glucose, when compared with that of control group [Figure 2b].

Further, the animals administered with *L. rhamnosus* showed few significant changes in the lipid profile. On day 14, the male animals administered with 1×10^8 CFU/mL of *L. rhamnosus* showed a significant change in non-HDL cholesterol when compared with that of the control group [Figure 3a] whereas female animals showed no significant changes in lipid profile when compared to the control group [Figure 3b]. On day 28, the male animals administered with 1×10^6 CFU/mL of *L. rhamnosus* showed a significant increase in the levels of LDL and total cholesterol/HDL

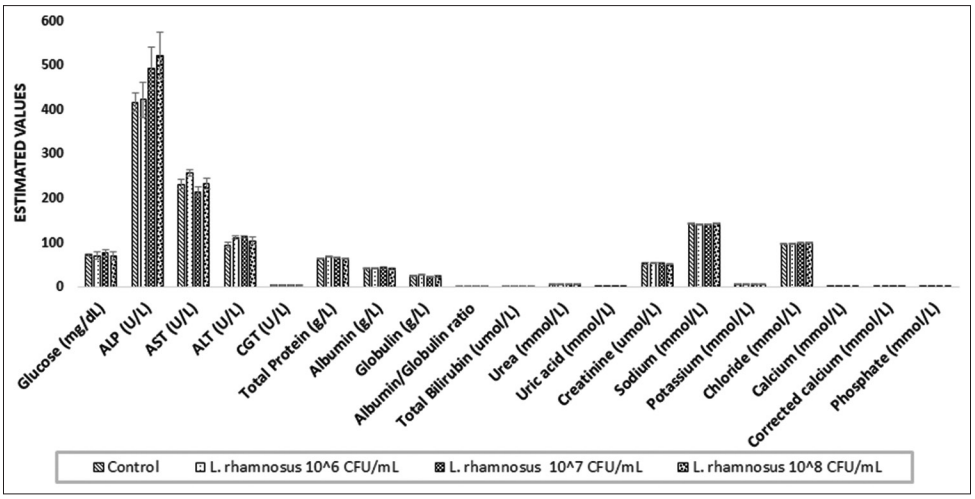

Figure 1a: Effect of *L. rhamnosus* on biochemcial parameters of male rats (14th day). All the values are mean ± SEM ($n = 6$).

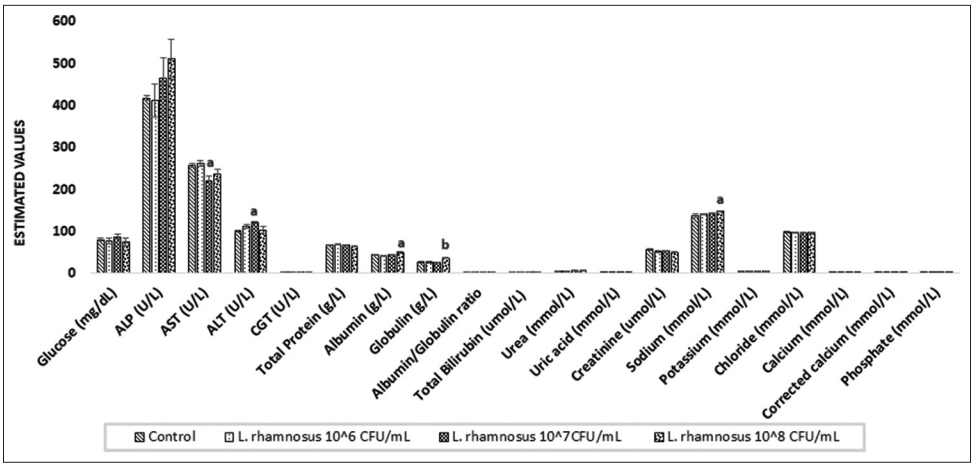

Figure 1b: Effect of *L. rhamnosus* on biochemical parameters of female rats (14th day). All the values are mean ± SEM ($n = 6$). $^{a}P<0.05$ and $^{b}P<0.01$ compared with the control (One-way ANOVA followed by Tukey's *post-hoc* test).

ratio when compared with that of the control group; male animals administered with 1×10^7 CFU/mL of *L. rhamnosus* showed a significant increase in the levels of LDL, non-HDL cholesterol and total cholesterol/HDL ratio when compared with that of the control group; male animals administered with 1×10^8 CFU/mL of *L. rhamnosus* showed a significant increase in the levels of LDL and non-HDL cholesterol when compared with that of the control group [Figure 4a]. Whereas female animals administered with 1×10^6 CFU/mL, 1×10^7 CFU/mL, and 1×10^8 CFU/mL of *L. rhamnosus* showed a significant increase in the levels of LDL, non-HDL cholesterol and total cholesterol/HDL ratio when compared with that of the control group [Figure 4b].

iii. Effect of *L. rhamnosus* on the haematological parameters

On day 14, the male animals administered with *L. rhamnosus* did not show any significant alterations in haematological parameters when compared with that of the control group [Table 1a]. Whereas the female animals showed significant alteration in the levels of RBC and MCHC in rats administered with 1×10^6 CFU/mL of *L. rhamnosus* when compared with that of the control group [Table 2a]. On day 28, the male animals administered with *L. rhamnosus* did not show any significant alterations in haematological parameters when compared with that of the control group [Table 1b], whereas female animals showed significant changes in PCV (1×10^6 CFU/mL), MCV (1×10^6 CFU/mL; 1×10^7 CFU/mL), MCHC (1×10^6 CFU/mL), RDW (1×10^7 CFU/mL; 1×10^8 CFU/mL) and WBC (1×10^6 CFU/mL) when compared with that of control group [Table 2b].

Effect of L. rhamnosus on the histopathology of organs

There has been no evidence of any histopathological abnormalities or changes observed in all the groups of animals [Figures 5 a-l]. In particular, no necrosis, fibrosis, loss of normal tissue architecture, adaptive changes–atrophy/hypertrophy, or inflammation was observed in any of the examined organs of experimental rats.

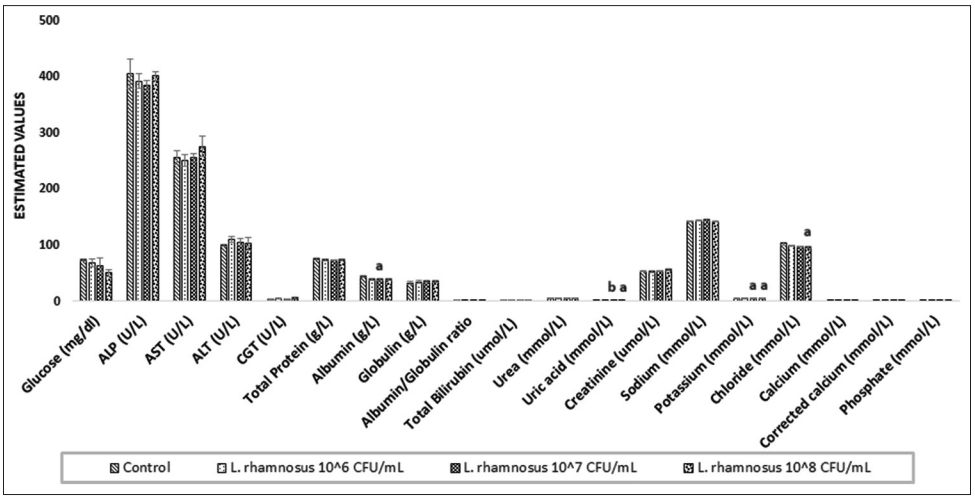

Figure 2a: Effect of *L. rhamnosus* on biochemical parameters of male rats (28th day). All the values are mean ± SEM ($n = 6$ except *L. rhamnosus* 1×10^8 CFU/mL administered group [$n=5$]). $^aP<0.05$ and $^bP<0.01$ compared with the control (One-way ANOVA followed by Tukey's *post-hoc* test)

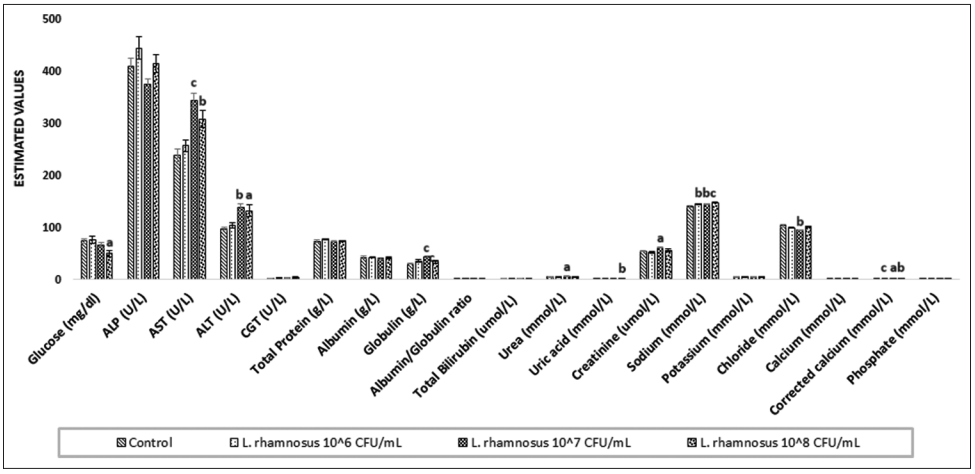

Figure 2b: Effect of *L. rhamnosus* on biochemical parameters of female rats (28th day). All the values are mean ± SEM ($n = 6$ except *L. rhamnosus* 1×10^8 CFU/mL administered group [$n=5$]). $^aP<0.05$, $^bP<0.01$ and $^cP<0.001$ compared with the control (One-way ANOVA followed by Tukey's *post-hoc* test).

Table 1a: Effect of *L. rhamnosus* on hematological parameters of male rats (14th day)

	Control	*L. rhamnosus* 1×10^6 CFU/mL	*L. rhamnosus* 1×10^7 CFU/mL	*L. rhamnosus* 1×10^8 CFU/mL
Hb (g/L)	126.83±6.67	124.17±9.94	144.17±3.57	141.33±2.42
RBC (/L)	$8.17\times10^{12}\pm1.86\times10^{11}$	$7.76\times10^{12}\pm3.72\times10^{11}$	$8.28\times10^{12}\pm1.90\times10^{11}$	$8.24\times10^{12}\pm9.42\times10^{10}$
PCV (L/L)	0.48±0.02	0.47±0.02	0.50±0.02	0.49±0.01
MCV (fL)	60.67±0.80	60.83±0.70	60.17±0.60	59.17±0.60
MCH (pg)	17.17±0.48	17.00±0.63	17.33±0.21	17.17±0.17
MCHC (g/L)	280.17±4.87	276.33±8.24	290.67±3.05	288.50±1.36
RDW (%)	17.28±0.42	17.25±0.75	17.60±0.51	17.70±0.44
WBC (/L)	$8.62\times10^9\pm3.71\times10^8$	$6.77\times10^9\pm1.51\times10^9$	$8.42\times10^9\pm4.91\times10^8$	$9.46\times10^9\pm8.03\times10^8$
Neutrophils (%)	22.62 ± 1.48	26.52 ± 1.39	25.31 ± 1.80	23.85 ± 2.96
Lymphocytes (%)	69.93 ± 1.39	64.66 ± 1.79	66.25 ± 1.51	68.53 ± 3.01
Monocytes (%)	5.13 ± 1.10	6.14 ± 1.33	5.47 ± 0.75	5.06 ± 0.93
Eosinophils (%)	2.32 ± 0.44	2.69 ± 0.58	2.97 ± 0.23	2.32 ± 0.14
Basophils (%)	0	0	0	0.25 ± 0.25
Platelets (/L)	$784\times10^9\pm400\times10^8$	$718\times10^9\pm116\times10^9$	$920\times10^9\pm147\times10^9$	$1060\times10^9\pm116\times10^9$

All the values mean ± SEM (n=6)

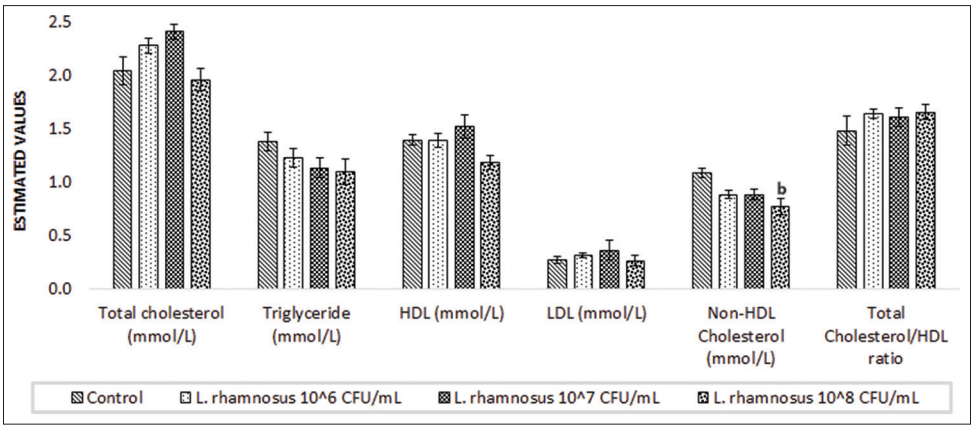

Figure 3a: Effect of *L. rhamnosus* on lipid profile of male rats (14th day). All the values are mean ± SEM ($n = 6$). $^{b}P<0.01$ compared with the control (One-way ANOVA followed by Tukey's *post-hoc* test).

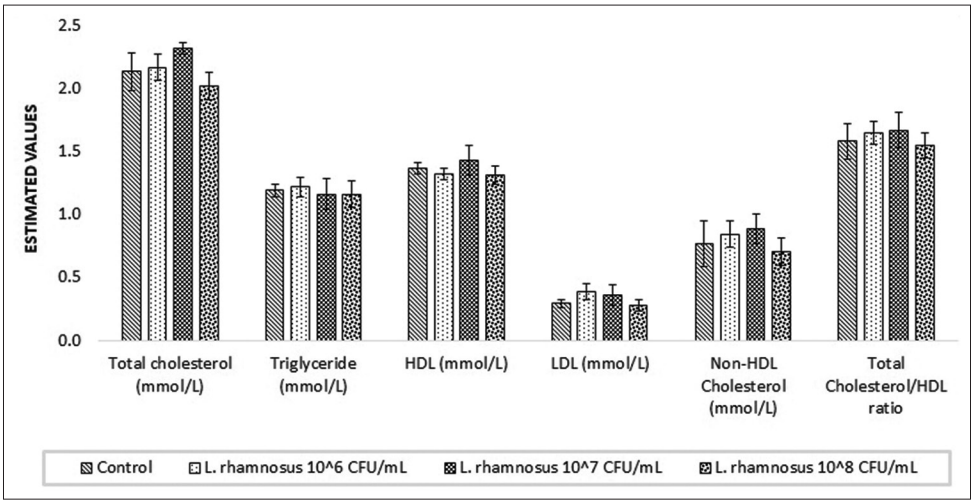

Figure 3b: Effect of *L. rhamnosus* on lipid profile of female rats (14th day). All the values are mean ± SEM ($n = 6$).

Table 1b: Effect of *L. rhamnosus* on hematological parameters of male rats (28th day)

	Control	*L. rhamnosus* 1×10^6 CFU/mL	*L. rhamnosus* 1×10^7 CFU/mL	*L. rhamnosus* 1×10^8 CFU/mL
Hb (g/L)	131.17±5.34	125.33 ± 13.56	136.67±3.36	135±1.10
RBC (/L)	$8.06\times10^{12}\pm1.05\times10^{11}$	$7.44\times10^{12}\pm8.22\times10^{11}$	$7.91\times10^{12}\pm1.66\times10^{11}$	$8.06\times10^{12}\pm1.30\times10^{11}$
PCV (L/L)	0.51±0.01	0.49±0.05	0.51±0.01	0.51±0.01
MCV (fL)	61.83±0.48	64.67±1.02	64.33±0.56	62.60±0.93
MCH (pg)	17.50±0.22	17.33±0.33	17.17±0.17	16.80±0.37
MCHC (g/L)	263.67±7.02	265.17±4.01	268.17±1.05	267.20±3.97
RDW (%)	17.33±0.62	18.03±0.66	18.25±0.34	18.68±0.54
WBC (/L)	$7.27\times10^9\pm1.32\times10^9$	$6.12\times10^9\pm1.39\times10^9$	$8.98\times10^9\pm1.56\times10^9$	$6.22\times10^9\pm2.67\times10^8$
Neutrophils (%)	27.56 ± 1.98	27.32 ± 1.87	25.36 ± 1.48	27.10 ± 2.67
Lymphocytes (%)	66.16 ± 2.95	66.29 ± 2.13	69.24 ± 1.61	65.72 ± 2.96
Monocytes (%)	3.64 ± 1.17	3.63 ± 0.55	3.19 ± 0.44	4.27 ± 0.83
Eosinophils (%)	2.65 ± 0.36	2.76 ± 0.59	2.21 ± 0.42	2.62 ± 0.48
Basophils (%)	0	0	0	0.29 ± 0.29
Platelets (/L)	$938\times10^9\pm119\times10^9$	$969\times10^9\pm198\times10^9$	$1150\times10^9\pm173\times10^9$	$1040\times10^9\pm933\times10^8$

All the values are mean ± SEM (n=6 except *L. rhamnosus* 10^8 CFU/mL [n=5]).

Discussion

Probiotics are an evolving approach in preventive therapy that is well-established in local and systemic infections in the human body.[12] Bacteriotherapy using this probiotic has

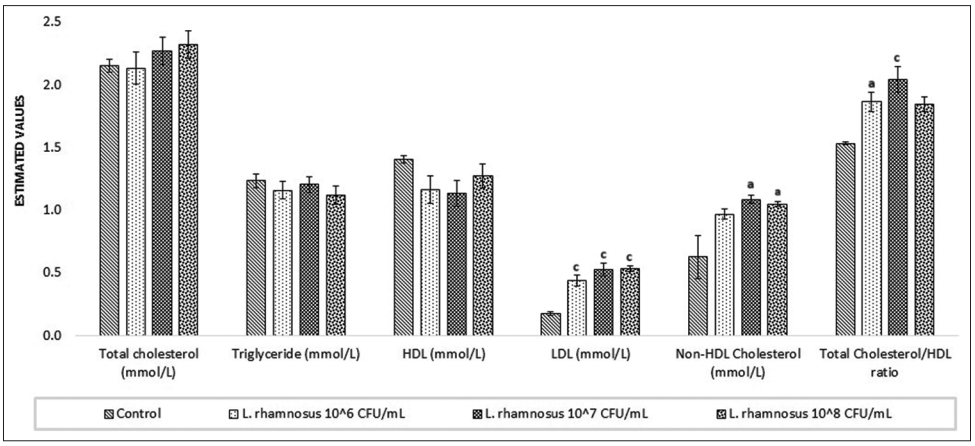

Figure 4a: Effect of *L. rhamnosus* on lipid profile of male rats (28th day). All the values are mean ± SEM (n = 6 except *L. rhamnosus* 1 × 10^8 CFU/mL administered group [n=5]). $^aP<0.05$ and $^cP<0.001$ compared with the control (One-way ANOVA followed by Tukey's *post-hoc* test).

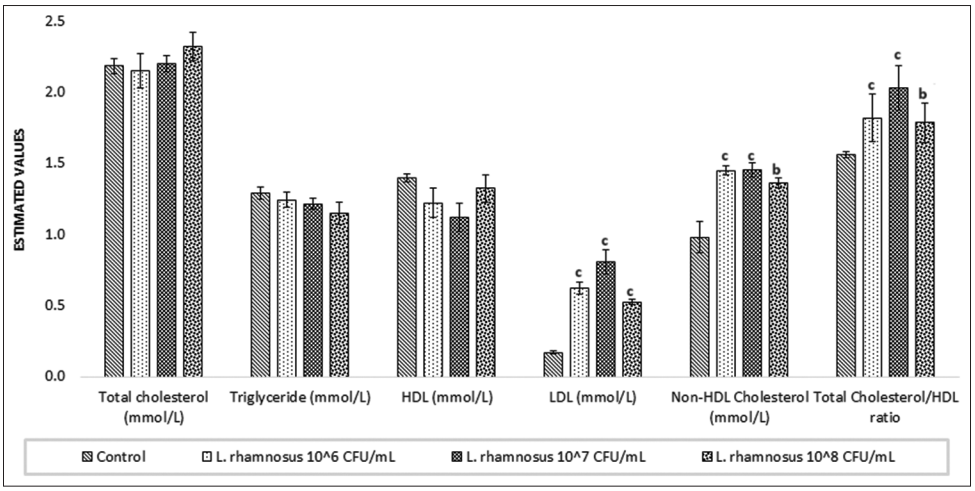

Figure 4b: Effect of *L. rhamnosus* on lipid profile of female rats (28th day). All the values are mean ± SEM (n = 6 except *L. rhamnosus* 1 × 10^8 CFU/mL administered group [n=5]). $^bP<0.01$ and $^cP<0.001$ compared with the control (One-way ANOVA followed by Tukey's *post-hoc* test).

become the most promising method for the prevention of several pathological diseases.[13,14] Among certified groups of probiotic bacteria, lactic acid bacteria have been considered safe bacteria with no obvious adverse effects.[15] However, the safety of *Lactobacillus* spp. as a probiotic has come into question due to recently reported unexpected reactions. These include bacteremia especially in immunocompromised patients and pediatric patients, bacteremia-associated endocarditis in cardiac patients, and contradictory results in pregnancy and lactation in ladies.[16,17] Among the various bacterial strains, *L. rhamnosus* strain is generally considered safe with minimum side effects.

Acute toxicity study provides primary toxicity information to establish the appropriate dose for future oral toxicity studies involving repeated doses.[18] Also, it evaluates the possible target organs for damage in sub-chronic toxicity studies in the future. Sub-chronic (repeated-dose oral toxicity) offers information into potential health risks associated with frequent exposure over a relatively smaller duration (28 days).[19]

In vivo, toxicity tests are deemed mandatory to test the safety parameters of probiotic bacteria under study before their commercial use in humans.[20] In the present acute oral toxicity study, a single dose of 1 × 10^{10} CFU/mL of *L. rhamnosus* did not show any mortality or any other evidence of toxicity in the experimental animals. Hence, a 28-day repeated-dose sub-chronic oral toxicity study with three dose levels of 1 × 10^6, 1 × 10^7 and 1 × 10^8 CFU/mL of *L. rhamnosus* was conducted to further explore their safety profiles. In the present study, toxicological variation within acceptable biological ranges was noted between the groups. These parameters included body weight, feed intake, general and behavioural characteristics, haematological and biochemical parameters.

The present study shows no observed adverse reactions and a steady increase in body weight after consumption of probiotics of *L. rhamnosus* strains. Body weight showed significantly higher values in all experimental groups when compared with that of the control. In animal studies, feed consumption and body weight serve as reliable indicators of adverse effects.

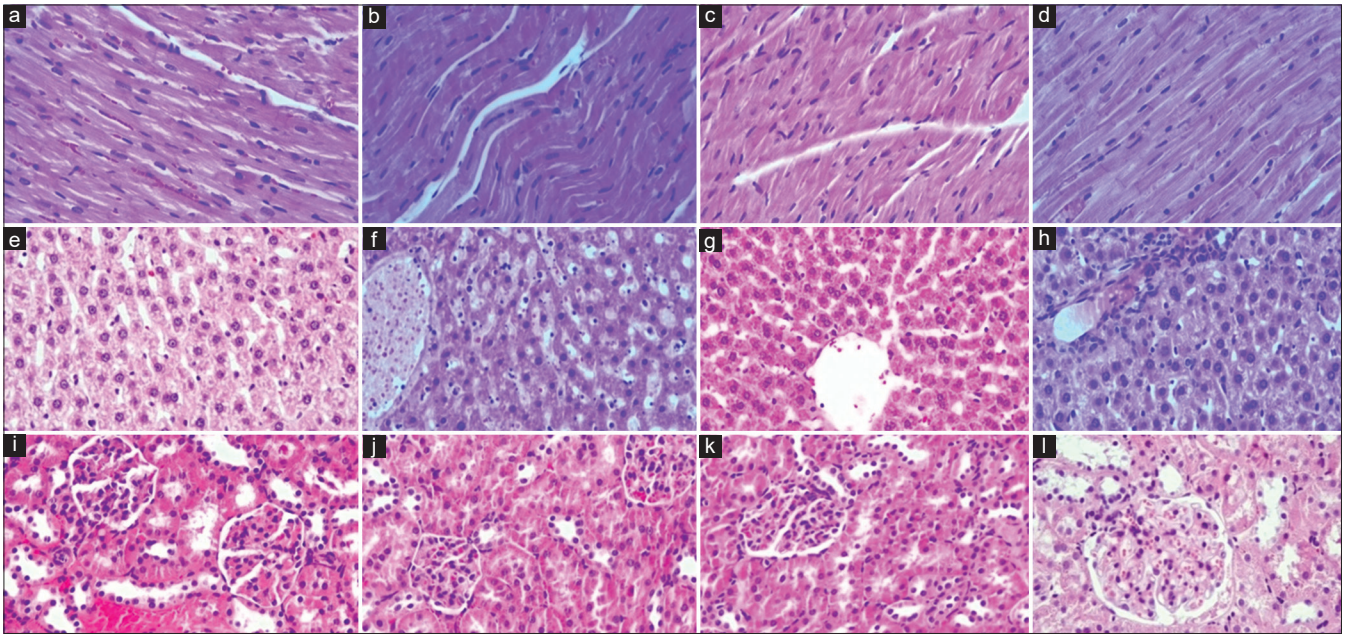

Figure 5: (a,e,i): Histological features of heart, liver and kindey of control rats; (b,f,j): Histological features of heart, liver and kindey of rats administered with *L. rhamnosus* 1×10^{6} CFU/mL; (c,g,k): Histological features of heart, liver and kindey of rats administered with *L. rhamnosus* 1×10^{7} CFU/mL and, (d,h,l): Histological features of heart, liver and kindey of rats administered with *L. rhamnosus* 1×10^{8} CFU/mL (H&E; 200x magnification)

Table 2a: Effect of *L. rhamnosus* on hematological parameters of female rats (14th day)

	Control	*L. rhamnosus* 1×10^{6} CFU/mL	*L. rhamnosus* 1×10^{7} CFU/mL	*L. rhamnosus* 1×10^{8} CFU/mL
Hb (g/L)	120.00±6.11	125.00±9.89	141.33±3.91	143.33±3.54
RBC (/L)	$8.25\times10^{12}\pm2.45\times10^{11}$	$6.10\times10^{12}\pm5.91\times10^{11}$***	$8.42\times10^{12}\pm9.97\times10^{10}$	$8.38\times10^{12}\pm1.43\times10^{11}$
PCV (L/L)	0.48±0.01	0.52±0.04	0.55±0.02	0.44±0.02
MCV (fL)	63.83±1.78	63.17±0.95	62.50±0.85	64.67±0.61
MCH (pg)	16.50±0.50	16.33±0.71	16.33±0.21	16.67±0.61
MCHC (g/L)	316.00±16.27	269.33±8.65*	344.83±3.12	288.67±2.08
RDW (%)	17.55±0.44	18.48±0.79	17.03±0.60	17.40±0.26
WBC (/L)	$7.59\times10^{9}\pm6.67\times10^{8}$	$7.12\times10^{9}\pm1.41\times10^{9}$	$7.98\times10^{9}\pm6.05\times10^{8}$	$9.58\times10^{9}\pm5.68\times10^{8}$
Neutrophils (%)	26.81 ± 3.12	23.41 ± 2.01	24.15 ± 1.13	24.43 ± 2.79
Lymphocytes (%)	65.91 ± 3.08	68.72 ± 2.97	69.33 ± 1.16	68.57 ± 3.01
Monocytes (%)	4.69 ± 0.27	5.73 ± 1.18	3.02 ± 0.38	4.92 ± 0.22
Eosinophils (%)	2.59 ± 0.20	2.14 ± 0.42	3.51 ± 0.46	2.08 ± 0.46
Basophils (%)	0	0	0	0
Platelets (/L)	$881\times10^{9}\pm326\times10^{8}$	$906\times10^{9}\pm274\times10^{8}$	$933\times10^{9}\pm123\times10^{9}$	$1100\times10^{9}\pm112\times10^{9}$

All the values are mean±SEM (n=6). *P<0.05 and ***P<0.001 compared with the control (One-way ANOVA followed by Tukey's *post-hoc* test)

Normally, this weight gain is attributed to very little variation in the body weight among the animals.[21]

In this study, there were no variances in the relative weights of brain, heart, lung, liver, spleen, kidney, adrenal, and gonadal organs among the experimental groups, and in comparison, with the controls. This indicated that the *L. rhamnosus* strains did not cause any notable toxicity as organ weight assessment is considered to be one of the indicators of acute pathology assessed in these toxicity tests.

In the present study, blood samples of the experimental rats were analyzed for lipid profile and biochemical parameters on the 14th and 28th day of administration of *L. rhamnosus*. Serum biochemical parameters analyzed in our study under lipid profile are good indicators of heart disease. Both male and female experimental rats showed significant changes in LDL, non-HDL, and total cholesterol values at varying degrees of *L. rhamnosus* dosage administration but under physiological limits suggesting no evidence of major cardiac changes. Alternatively in our study, the alteration in ALP, AST, and ALT may indicate liver damage. The vicious cycle of electrolytes (sodium, potassium, chloride, and calcium) balanced with creatinine, uric acid, and urea is essential to overcome any changes associated with renal failure.[22] Although the above parameters showed statistical significance, they are within the physiological limit of deviation as evidenced and confirmed further on histopathological examination (absence of any pathology) in the heart, liver,

Table 2b: Effect of *L. rhamnosus* on hematological parameters of female rats (28th day)

	Control	*L. rhamnosus* 1×10^6 CFU/mL	*L. rhamnosus* 1×10^7 CFU/mL	*L. rhamnosus* 1×10^8 CFU/mL
Hb (g/L)	131.83±5.18	137.33±9.87	139.50±4.07	125.80±1.20
RBC (/L)	$8.14\times10^{12}\pm2.53\times10^{11}$	$7.69\times10^{12}\pm7.27\times10^{11}$	$8.17\times10^{12}\pm7.99\times10^{10}$	$8.39\times10^{12}\pm1.23\times10^{11}$
PCV (L/L)	0.52±0.01	0.42±0.03*	0.49±0.02	0.46±0.02
MCV (fL)	61.17±0.54	66.00±1.10**	64.67±0.56*	63.80±0.73
MCH (pg)	17.33±0.21	17.33±0.33	17.33±0.21	17.20±0.37
MCHC (g/L)	274.33±5.14	257.33±3.54*	275.83±1.56	264.80±2.50
RDW (%)	16.30±0.53	17.85±0.42	18.20±0.36*	20.40±0.62***
WBC (/L)	$5.62\times10^9\pm6.72\times10^8$	$8.82\times10^9\pm7.87\times10^{8*}$	$8.37\times10^9\pm1.10\times10^9$	$6.84\times10^9\pm4.47\times10^8$
Neutrophils (%)	27.90 ± 3.08	27.71 ± 2.75	27.35 ± 2.15	29.08 ± 2.04
Lymphocytes (%)	63.96 ± 3.74	64.40 ± 2.71	64.27 ± 2.76	63.91 ± 2.15
Monocytes (%)	4.52 ± 0.50	4.67 ± 0.49	4.39 ± 0.80	4.84 ± 0.56
Eosinophils (%)	3.62 ± 0.89	3.22 ± 0.55	3.99 ± 0.90	2.17 ± 0.51
Basophils (%)	0	0	0	0
Platelets (/L)	$888\times10^9\pm557\times10^8$	$921\times10^9\pm106\times10^9$	$1190\times10^9\pm140\times10^9$	$1120\times10^9\pm689\times10^8$

All the values are mean±SEM (n=6 except *L. rhamnosus* 10^8 CFU/mL [n=5]). *P<0.05, **P<0.01 and ***P<0.001 compared with the control (One-way ANOVA followed by Tukey's *post-hoc* test)

and kidney. The notable reduction in glucose levels seen with high doses of *L. rhamnosus* may be due to its substantial impact on insulin resistance by reduction of blood glucose level through downregulation of gluconeogenesis gene expression.[23] Serum biochemical analysis is critically vital in discerning toxicological effects induced by treatments and identifying organ-related issues. In the present study, only a few haematological parameters showed statistically significant changes. Haematological parameters are usually used for the determination of inflammation and infections.

Bhat *et al*. conducted similar studies to assess the safety factor of *L. rhamnosus* MTCC-5897 administered for a cycle of 28-day period on the mice. This study also concluded that doses in the range of 10^7, 10^9, 10^{11}, and 10^{13} CFU/mL/day on administration to the mice showed no adverse reactions on the animal body weight, organ weight indices, and clinical chemistry parameters. Bhat *et al*. also noticed reduced values in ALT which were under normal biological limits suggestive of normal liver and kidney function considering it to be a safe food additive.[24]

Zhang *et al*. also conducted a similar study to assess the safety of *L. rhamnosus* MP108 for 90 days on the SD rats with a dose range of 0.25, 0.50, and 1.50 g/kg/bw/day.[25] No adverse reactions were noticed throughout the study. Adding to this, no statistically notable changes were seen in the haematological, biochemical, urinary, and pathological parameters indicative of its safe use in the food supplements.

Steele, reviewed the clinical use and efficacy of *L. rhamnosus* on various oral and systemic diseases in humans.[26] It was evident that *L. rhamnosus* is considered safe and it is used as a suitable probiotic with its antimicrobial, anti-inflammatory, and immunomodulatory activities for several diseases like gastrointestinal, respiratory, skin diseases, and dental caries in pediatric and geriatric patients. Overall, the present study suggested no evidence of any fatal toxicologically mediated inflammation or infection.

Apart from the studies that have been mentioned above that have tested the safety assessment of only *L. rhamnosus* single strain on the rats, Zhou *et al*., in 2000 and Bernardeau *et al*., conducted *in vivo* safety assessment of *L. rhamnosus* strain along with other *Lactobacillus* strain at repeated doses on rats for a period of 4 weeks. No significant toxicological changes were noted on the rats even after consumption of this probiotic ranging to a maximum of 10^{12} CFU/kg bw/day and 10^8 CFU/mouse/day, respectively.[27,28]

Conclusion

In the present study, probiotic *L. rhamnosus* exhibited mild-to-moderate toxic effects at the dose levels of 1×10^6, 1×10^7 and 1×10^8 CFU/mL in rats. While the haematological and biochemical parameters assessed were statistically significant, they remained under physiological limits, and there was no evidence of histopathological changes in heart, liver and kidneys. These findings support the *L. rhamnosus* is safe for both preventive and therapeutic applications in the management of pathological diseases in future.

Financial support and sponsorship

We are extremely thankful to the Research Management Centre, AIMST University for supporting our research with the postgraduate fund.

Conflicts of interest

There are no conflicts of interest.

References

1. Joint FAO/WHO Expert consultation on evaluation of health and nutritional properties of probiotics in food including powder milk with live Lactic Acid Bacteria, 1-4 October 2001.
2. Martín R, Langella P. Emerging health concepts in the probiotics field: Streamlining the definitions. Front Microbiol 2019;10:1047.
3. Hill C, Guarner F, Reid G, Gibson GR, Merenstein DJ, Pot B, *et al*. The international scientific association for probiotics and Prebiotics consensus statement on the scope and appropriate use of the term probiotic. Nat Rev Gastroenterol Hepatol 2014;11:506-14.

4. Chugh P, Dutt R, Sharma A, Bhagat N, Dhar MS. A critical appraisal of the effects of probiotics on oral health. J Funct Foods 2020;70:1-10.
5. Ayivi RD, Gyawali R, Krastanov A, Aljaloud SO, Worku M, Tahergorabi R, *et al*. Lactic acid bacteria: Food safety and human health applications. Dairy 2020;1:202-32.
6. Duche RT, Singh A, Wandhare AG, *et al.* Antibiotic resistance in potential probiotic lactic acid bacteria of fermented foods and human origin from Nigeria. BMC Microbiol 2023; 23: 142.
7. Hill D, Sugrue I, Tobin C, Hill C, Stanton C, Ross RP. The *Lactobacillus casei* Group: History and health related applications. Front Microbiol 2018;9:1-12.
8. Petrariu OA, Barbu IC, Niculescu AG, Constantin M, Grigore GA, Cristian RE, *et al*. Role of probiotics in managing various human diseases, from oral pathology to cancer and gastrointestinal diseases. Front Microbiol 2024;14:1296447.
9. Agamennone V, Krul CAM, Rijkers G, Kort R. A practical guide for probiotics applied to the case of antibiotic-associated diarrhea in The Netherlands. BMC Gastroenterol 2018;18,103.
10. OECD. Test no. 423: Acute oral toxicity-acute toxic class method. OECD Guidelines for the Testing of Chemicals, Section 4 Paris: OECD Publishing; 2002.
11. OECD. Test no. 407: Repeated dose 28-day oral toxicity study in rodents. OECD Guidelines for the Testing of Chemicals, Section 4. Paris: OECD Publishing.
12. Li X, Wang Q, Hu X, Liu W. Current status of probiotics as supplements in the prevention and treatment of infectious diseases. Front Cell Infect Microbiol 2022;14;12:789063.
13. Milner E, Stevens B, An M, Lam V, Ainsworth M, Dihle P, *et al*. Utilizing probiotics for the prevention and treatment of gastrointestinal diseases. Front Microbiol 2021;12:689958.
14. Aponte M, Murru N, Shoukat M. Therapeutic, prophylactic, and functional use of probiotics: A current perspective. Front Microbiol 2020;11:562048.
15. Liu, C, Xue WJ, Ding H, An C, Ma SJ, Liu Y. Probiotic potential of Lactobacillus strains isolated from fermented vegetables in Shaanxi, China. Frontiers in microbiology 2022; 12: 774903.
16. Rahman A, Alqaisi S, Nath J. A case of lactobacillus casei endocarditis associated with probiotic intake in an immunocompromised patient. Cureus 2023;15:e38049.
17. Kullar R, Goldstein EJC, Johnson S, McFarland LV. *Lactobacillus* bacteremia and probiotics: A review. Microorganisms 2023;11:896.
18. Kojima H, Nakada T, Yagami A, Todo H, Nishimura J, Yagi M, *et al*. A step-by-step approach for assessing acute oral toxicity without animal testing for additives of quasi-drugs and cosmetic ingredients. Curr Res Toxicol 2022;4:100100.
19. Kpemissi M, Metowogo K, Melila M, Veerapur VP, Negru M, Taulescu M, *et al*. Acute and subchronic oral toxicity assessments of *Combretum micranthum* (Combretaceae) in Wistar rats. Toxicol Rep 2020;18;7:162-8.
20. Shokryazdan P, Faseleh Jahromi M, Liang JB, Kalavathy R, Sieo CC, Ho YW. Safety assessment of two new *Lactobacillus* strains as probiotic for human using a rat model. PLoS One 2016;11:e0159851.
21. Parasuraman S. Toxicological screening. J Pharmacol Pharmacother 2011;2:74-9.
22. McGregor and Jones. Fluid and electrolyte problems in renal dysfunction. Anaesth Intensive Care. 2021; 22: 406-409.
23. Farida E, Nuraida L, Giriwono PE, Jenie BSL. *Lactobacillus rhamnosus* Reduces Blood Glucose Level through downregulation of gluconeogenesis gene expression in streptozotocin-induced diabetic rats. Int J Food Sci. 2020;2020:6108575.
24. Bhat MI, Singh VK, Sharma D, Kapila S, Kapila R. Adherence capability and safety assessment of an indigenous probiotic strain *Lactobacillus rhamnosus* MTCC5897. Microb Pathog. 2019;130:12030.
25. Zhang B, Lynch B, Zhao J, Guo Y, Mak A. *Lactobacillus rhamnosus* MP108: Toxicological evaluation. J Food Sci. 2021;86:22841.
26. Steele C. *Lactobacillus rhamnosus* GG: A review of clinical use and efficacy. Nutr Med J. 2022;1:70116.
27. Zhou JS, Shu Q, Rutherfurd KJ, Prasad J, Birtles MJ, Gopal PK, *et al*. Safety assessment of potential probiotic lactic acid bacterial strains *Lactobacillus rhamnosus* HN001, Lb. acidophilus HN017, and Bifidobacterium lactis HN019 in BALB/c mice. Int J Food Microbiol 2000;56:87-96.
28. Bernardeau M, Vernoux JP, Gueguen M. Safety and efficacy probiotic lactobacilli in promoting growth in postweaning swiss mice. Int J Food Microbiol 2002;77:1927.